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Short Paper

Bacillus licheniformis isolated during a fish kill is non-pathogenic

David J PASNIK,[1]* Joyce J EVANS[1] AND Phillip H KLESIUS[2]

[1]*Aquatic Animal Health Research Laboratory, United States Department of Agriculture – Agricultural Research Service, Chestertown, MD 21620 and* [2]*Aquatic Animal Health Research Laboratory, United States Department of Agriculture – Agricultural Research Service, Auburn, AL 36832, USA*

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Some fish populations in Chesapeake Bay, USA have been reportedly declining in recent years.[1–3] Considerable effort is being made to determine factors associated with this population decline, although the mechanisms have not been fully clarified.[4,5] Significant mortalities among fish in the bay and its tributaries have sometimes been attributed to pathogens including viruses,[6] harmful algae,[7–9] parasites,[10] fungal organisms,[4] bacteria[11,12] or a combination thereof.[13] When a fish kill occurred in October 2006 in the Corsica River, a tributary of Chesapeake Bay located near Centreville, MD, USA, the event affected mostly white perch *Morone americana* and Atlantic menhaden *Brevoortia tyrannus*. The fish kill lasted 6 days, and resulted in approximately 1800 fish mortalities subsequently associated with periods of low oxygen, increased ammonia levels and increased activity of *Karlodinium veneficum*.[14,15] Given that bacterial organisms have also played significant roles in other Maryland waterway fish kills, a study was performed to determine whether bacteria contributed to the significant mortalities.

Several other species of fish, including gizzard shad *Dorosoma cepedianum*, yellow perch *Perca flavescens* and striped bass *Morone saxatilis*, were noted floating on the water surface at the sampling sites. A cast net was used to sample live white perch ($n = 7$, mean weight = 15.6 g) and Atlantic menhaden ($n = 7$, mean weight = 18.2 g) at fish kill sites in the river. Dead fish captured and examined in the area exhibited an advanced state of autolysis, so no dead fish were used in this study. The approximate mean water quality parameters at the sampling sites were: temperature 20.22°C, dissolved oxygen 8.26 mg/L, salinity 7.6 and pH 8.36. Blood glucose at the time of capture was measured using the methods of Evans *et al.*[16] and ranged from 53 to 216 mg/dL, with a mean of 95.0 ± 56.6 mg/dL. The fish were transported in a cooler with ambient river water and necropsied at the Aquatic Animal Health Research Laboratory (AAHRL) in Chestertown, MD, USA. The fish were kept in 57-L aquaria supplied with flow-through dechlorinated tap water. Solar salt (NaCl, Cargill, Minneapolis, MN, USA) was added to maintain approximately 1.0 salinity, and the fish were maintained on a 12 h:12 h light–dark period at approximately 25°C. The menhaden did not thrive in the aquaria possibly due to exposure to stressors caused by transportation, unresponsiveness to pelleted feed, disruption of normal schooling in the rectangular tanks and/or crowding of fish in the tank corners. Mortalities occurred soon after placement in the aquaria, and dead fish were promptly removed while moribund fish were euthanized with MS-222 (Argent, Redman, WA, USA). All menhaden died or were euthanized within 7 days of arrival at the laboratory, and all menhaden were frozen at –20°C before microbiological analysis.

None of the sampled wild white perch or menhaden exhibited gross external or internal lesions at necropsy, and no indication of viral, parasitic or fungal disease was noted. Samples for microbiological examination were aseptically obtained from the nares and intestine of all white perch and the nares, brain, head kidney, intestine and posterior kidney of all menhaden using sterile Remel Bacti-Swab NPG collection and transport systems (Remel, Lenexa, KS, USA). The samples were sent in transport tubes with unbroken media ampoules to the laboratory in Auburn, AL, USA within 1 day. Evans *et al.*[17] indicated that unbroken media ampoules could optimize the viability of bacteria. The transported swabs were used to inoculate 5 mL of tryptic soy broth (TSB, Difco, Detroit, MI, USA) enriched with defibrinated sheep blood. The

*Corresponding author: Tel: 1-410-778-4136. Fax: 1-410-778-4399. Email: david.pasnik@ars.usda.gov
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inoculated TSB was incubated for 3–4 h at 27°C, and broth samples were then streaked on 5% sheep blood agar (Remel) and incubated for 18–24 h at 27°C.

No bacterial isolates were obtained from the white perch. Twelve bacterial isolates were obtained from five of the seven menhaden, with isolation of bacteria from the intestines (seven isolates), nares (four) and liver (one). Four of these isolates formed flat, irregularly shaped white vegetative colonies that were variably mucoid and rough, and the colonies consisted of spore-forming, Gram positive, oxidase-positive and catalase-positive rods. Pure colonies were then sampled and subjected to morphological and biochemical tests for phenotypic characterization.[18] The fatty acid methyl ester (FAME) profile of the isolates was analyzed with the Microbial Identification System (MIDI, Newark, DE, USA) as described in Panangala *et al.*[19] *Bacillus licheniformis* was isolated from either the nares or intestines of four fish as identified to the species level by FAME analysis [similarity index (SI) ranged 0.376–0.496]. In addition, of the 12 isolates, one isolate each of *Legionella brunensis* (SI = 0.530, isolated from the intestines), *Corynebacterium diphtheriae gravis* (SI = 0.249, nares), and *Bacillus thuringiensis* (SI = 0.251, intestines) was each cultured from different fish and identified by FAME. Five isolates could not be definitively identified.

A challenge experiment with one *B. licheniformis* isolate [US Department of Agriculture (USDA) #06-FKCO-AM-13INT] was conducted using Nile tilapia *Oreochromis niloticus* because: (i) these fish are available at the AAHRL; (ii) there are difficulties holding menhaden in available aquaria; and (iii) there are reported beneficial effects of *B. licheniformis* as a probiotic in Nile tilapia.[20] The Nile tilapia ($n = 60$, mean weight $= 26.0 \pm 0.8$ g) were acclimated in the AAHRL facility for one year, placed into 57-L aquaria, supplied with flow-through dechlorinated tap water, and maintained on a 12 h:12 h light–dark photoperiod. The fish were fed daily to satiation with Aquamax Grower (Aquamax, Brentwood, MO, USA). The isolate was initially held in a –78°C freezer, was not passed through other fish before challenge, and was grown at 22°C for 24 h on tryptic soy agar (TSA, Difco) to prepare the challenge isolate. Isolate identity was confirmed as *Bacillus* sp. using the Biolog MicroLog3 Microbial Identification System (Biolog, Hayward, CA, USA) according to the manufacturer's instructions. Initial culture was performed using the 'Special procedures for spore-forming Gram positive rods' protocol, which included culture on Biolog Universal Growth (BUG)–maltose–thioglycolate agar and identification by the MicroLog and User databases. Biolog identified the isolate to the genus level as *Bacillus* sp. (SI = 0.48, Distance = 7.34). Isolate identity was also confirmed by tDNA–polymerase chain reaction fingerprinting, using the methods of Borin *et al.*[21] with slight modifications. The test resulted in signature agarose gel bands of 140, 160 and 750 bp, indicating *B. licheniformis* identity.

The fish were challenged by intraperitoneal injection with 1.0×10^8, 10^7, 10^6, 10^5 or 10^4 colony forming units of *B. licheniformis* per fish or sham-challenged with TSB, sequestered as 10 fish/tank according to dose group, and observed for 7 days. Fish were monitored daily for clinical signs of disease and mortality, and moribund and dead fish were promptly removed. Daily water temperature for the challenged tilapia averaged 20.8 ± 0.05°C, mean daily dissolved oxygen was 5.0 ± 0.17 mg/L and mean daily total ammonia concentration was 0.38 ± 0.03 mg/L.

None of the tilapia in the challenge study exhibited clinical signs of disease, and no external or internal lesions were noted. There were no mortalities after challenge. At the end of the study 7 days postchallenge, fish were euthanized with MS-222. Samples for microbiological examination were aseptically obtained from the nares, brain, head kidney, intestine and posterior kidney of the fish to isolate *B. licheniformis*; samples were cultured at 22°C for 24 h on TSA. No fish were sampled for bacteriology before the end of the 7-day challenge. No *B. licheniformis* was isolated from sampled fish at the termination of the challenge study. Given that the bacteria could not be re-isolated from the fish, the isolate could not be sequentially passed through fish to increase its virulence. Given that no morbidity or mortality was elicited with the experimental challenge, the findings in this study do not suggest that *B. licheniformis* was a primary causative agent of the Corsica River fish mortalities and can not provide evidence that *B. licheniformis* is pathogenic to fish.

Other studies may indirectly confirm this suggestion because *B. licheniformis* has been used as a beneficial probiotic to increase: (i) survival of Nile tilapia *Oreochromis niloticus*;[20] (ii) resistance of rainbow trout *Oncorhynchus mykiss* against *Yersinia ruckeri*;[22] and (iii) growth of crucian carp *Carassius carassius*.[23] *Bacillus licheniformis* may also be normal flora of some fish species.[22,23] Further, since *B. licheniformis* is ubiquitous in the environment and is used in sewer treatment plants often voided into rivers like the Corsica River,[18] it would be fortunate that the bacterial species is non-pathogenic to fish. Other researchers have examined whether *B. licheniformis* is pathogenic to human and animals, and such studies have gener-

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ally indicated that the bacteria presents low risk to mammalian health or is non-pathogenic except in immunocompromized individuals or secondary infections.[18,24] Given the periods of low oxygen and increased ammonia levels and the presence of *K. veneficum* in the Corsica River,[14,15] as well as the presumptively increased blood glucose levels in some of the sampled menhaden, it is possible that the fish may become stressed and subsequently immunocompromized. Although not proven here, this could make them more susceptible to disease caused by *Bacillus* spp. Further experiments would be required to definitively determine the environmental factors that increase susceptibility to *Bacillus* spp.

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